Characterization of antibody-antigen interactions by fluorescence spectroscopy

Book Section

25

Characterization of Antibody-Antigen Interactions by Fluorescence Spectroscopy

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1. Introduction

Fluorescence spectroscopy is a widely used technique for the characterization of molecular interactions in biological systems. It is highly sensitive, and allows measurements at low sample concentrations (down to 10^{-7} *M*). It is also available at a reasonably low equipment cost. These features make fluorescence spectroscopy an attractive technique as compared to other forms of optical spectroscopy.

Fluorescence is a three-stage process that occurs in certain molecules known as fluorophores or fluorescent dyes (generally polyaromatic hydrocarbons or heterocycles). The process leading to fluorescence emission from such molecules is illustrated by the Jablonski diagram **(Fig. 1)**. The first stage is the excitation step, in which the fluorophore absorbs a photon of energy $h\nu_{EX}$ and becomes an excited electronic singlet state (S_1'). The excitation wavelength is usually the same as the absorption wavelength of the fluorophore. The second stage is the excited-state lifetime, when the excited state exists for a finite period of time (typically $1–10 \times 10^{-9}$ s). During this period, the fluorophore undergoes conformational changes and is also subject to possible interactions with its molecular environment, with two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (stage 1) return to the ground state (S_0) by fluorescence emission, as other processes such as collisional quenching, fluorescence energy transfer, and intersystem crossing may also depopulate S_1. The third stage is fluorescence emission, when a photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Because of energy dissipation during the excited-state lifetime, the energy of this photon is lower, and thus of

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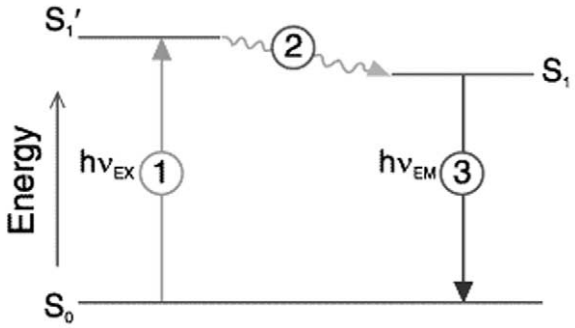

Fig. 1. The Jablonski diagram illustrates the three stages involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence.

a longer wavelength, than the excitation photon $h\nu_{EX}$. Consequently, the ratio of the number of fluorescence photons emitted (stage 3) to the number of photons absorbed (stage 1) represents the *fluorescence quantum yield.*

The fluorescence process recurs, and the same fluorophore can be repeatedly excited and detected. For polyatomic molecules in solution, the discrete electronic transitions represented by $h\nu_{EX}$ and $h\nu_{EM}$ in **Fig. 1** are replaced by broad energy spectra known as the fluorescence excitation and fluorescence emission spectrum, respectively. With few exceptions, the excitation spectrum of a single species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, because of the partial dissipation of excitation energy during the excited-state lifetime. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength. Fluorescence emission intensity depends on the same parameters as absorbance—defined by the Beer-Lambert law as the product of the molar extinction coefficient, optical pathlength, and concentration—as well as on the fluorescence quantum yield, the intensity of the excitation source, and the efficiency of the instrument and, in dilute solutions, is linearly proportional to these parameters.

Fluorescence is an absorption/emission phenomenon that has been associated with a number of biological molecules and extensively reviewed in the literature ***(1)***. The induction of fluorescence is determined by the presence of certain chromophores within a molecule. When examining proteins, these chromophores are found in the aromatic amino acids tryptophan, tyrosine, and phenylalanine, which have high densities of delocalized electrons. Of the three aromatic amino acids, tryptophan has by far the greatest contribution to the flu-

orescence of proteins. Its excitation wavelength is approx 295 nm. The emission wavelength varies according to the local environment of the tryptophans—e.g., 320 nm for buried residues, and 360 nm for solvent exposed residues.

Alterations in intensity of the tryptophan emission spectrum can be studied when an antibody is involved in a binding event. A reduction in the intensity of the fluorescence emission is observed upon binding of the antibody to its antigen. However, such a reduction is dependent upon the presence of tryptophan residues in the vicinity of the antibody binding-pocket that would make them susceptible to the minor alterations in the antibody structure upon binding, thus resulting in the reduction of their fluorescence emission. This is known as fluorescence quenching, and is especially useful when the binding partner, the antigen, contains no fluorescent chromophores and is thus invisible to the fluorimeter. When studying the intensity of the antibody fluorescent signal, the changes observed are solely caused by the change in the environment of the tryptophans within the antibody.

Two methods are described here for the study of antibody-antigen interactions using fluorescence quenching. The first method is based on keeping the antibody concentration constant and varying the antigen concentration accordingly to achieve various antibody-antigen ratios. This is an inverse titration, in which the first point measured is that of the antibody-antigen complex, when the antigen is in excess and maximum binding has been achieved. The antigen concentration is reduced at subsequent steps as aliquots of the antibody-antigen complex are replaced by aliquots of free antibody at the same concentration. A final point of free antibody in the absence of antigen is measured.

The second method is based on continuously increasing antigen concentrations throughout the titration. This method is a straightforward titration, in which the first point is that of free antibody, to which aliquots from a stock antigen solution are added until antigen is in excess in solution and saturation of all antigen-binding pockets of the antibody has been achieved. The data must then be normalized as the volume, and thus the concentration of the antibody changes every time an antigen aliquot is added to the solution. This method presents the significant advantage of using much smaller amounts of antibody material. However, normalizing data for volume addition has the potential to incorporate errors in a system that is so sensitive to volume changes. It is therefore imperative to know how sensitive your system is to changes in volume/concentration before you use this method of analysis. Each of the two methods therefore presents certain advantages and disadvantages (*see* **Notes 1** and **2**), which should be considered before an appropriate choice of method is made. Furthermore, both methods have a general applicability and can be used with other spectroscopic techniques for the analysis of molecular interactions, including those of antibody-antigen interactions (*see* **Note 3**).

The analysis of the data is sometimes as important as taking the experimental measurements. In the past, many workers have employed the Scatchard analysis plots for determining equilibrium association constants k_a. Scatchard analysis was first introduced ***(2)*** to provide data from straight-line plots produced for systems that were completely homogeneous and monomeric. An examination of most of the published uses of Scatchard plots makes it clear that many workers fit linear plots through nonlinear data. These situations oversimplify the binding event, and can incorporate large errors in the determination of binding constants. We therefore recommend (*see* **Note 4**) that users analyze their data using **Equation 1** ***(3,4)*** or variations of it using nonlinear least squares fitting, which would alleviate such problems of data fitting:

$$\mathrm{Acalc(P0)} = \left[\left[\frac{(\mathrm{E1}-\mathrm{E2})\left[(1+\mathrm{K}\cdot\mathrm{D}+\mathrm{K}\cdot\mathrm{P0})-\sqrt{(1+\mathrm{K}\cdot\mathrm{D}+\mathrm{K}\cdot\mathrm{P0})^2-4\cdot\mathrm{K}\cdot\mathrm{K}\cdot\mathrm{D}\cdot\mathrm{P0}}\right]}{2\cdot\mathrm{K}}\right]+\mathrm{E2}\cdot\mathrm{D}\right]\cdot\frac{1}{\mathrm{D}}$$

Equation 1: Quadratic equation for fitting binding curves.

where K is the equilibrium association constant, d is the concentration of antibody, E1 is the maximum value of Acalc(P0), and E2 is the ratio Fo/F at F equal to Fo (no antigen present) and therefore equals 1. Acalc(P0) is the calculated parameter representing F_o/F, the maximum fluorescence of the free antibody divided by the fluorescent signals obtained in the presence of quenching antigen. P0 is the total antigen concentration. Values of Fo/F for each antigen concentration vs antigen concentration at each titration point will have to be imported. Values of K and E1 are iteratively manipulated until the best fit of line to data is achieved or computer-calculated for best fit.

2. Materials

1. Phosphate-buffered saline (PBS): dissolve one PBS tablet (Sigma; cat. #P4417) in 200 mL of distilled water to give yield 0.01 *M* phosphate buffer, 0.0027 *M* KCl, and 0.137 *M* NaCl, pH 7.4; sterile-filter through a 0.22-µm filter.
2. Antibody of interest: store the antibody at –20°C in small aliquots to avoid denaturation or inactivation from repeated freeze-thaw cycles.
3. Antigen of interest: Depending on the nature of your antigen—if it is a small peptide, keep under dry conditions as solid and dissolve in buffer as needed, whereas if it is a protein in solution, also keep frozen in small aliquots to avoid denaturation or inactivation through repeated freeze-thaw cycles.
4. Measurements can be taken in any fluorimeter available in your laboratory, using fluorescence quartz cuvet. We have used a Perkin-Elmer LS-5 luminescence spectrometer.

3. Methods

3.1. Using Fixed Antibody Concentration

When using a fixed concentration of the antibody and varying concentrations of the antigen, one can produce a binding curve from which equilibrium constants K_A and K_D can be derived. This method was previously employed by Reedstrom et al. ***(5)*** for the study of protein-DNA interactions, but it can equally be applied to the study of antibody-antigen interactions as well as most intermolecular interactions. The method of using fixed antibody concentration is in fact a reverse titration, in which the initial measurement is of antibody fully saturated by antigen and the last is of free antibody.

1. Prepare in a fluorescence quartz cuvet a 2-mL solution of the antibody/antigen mixture. The initial concentration of the antibody should be 0.26 μ*M,* expressed in antigen-binding pockets (or 0.13 μ*M* of whole IgG molecules); the initial concentration of antigen epitopes should be 1.5 μ*M*. If a multimeric antigen is used, be sure to calculate the concentration per epitope and not for the whole antigen. It is generally believed that antibody-antigen interactions are straightforward, one-to-one interactions. However, this is not always the case, and care should be taken in calculating the concentration of antigen per epitope and of the antibody per antigen-binding pocket. Cooperativity effects can be observed with multimeric antigens with many epitopes, and this should be taken into consideration when calculating the association constants (*see* **Note 5**). The excess concentration of antigen epitopes (~5.8:1) ensures total saturation of all antigen-binding pockets on the antibody.
2. Prepare a separate free antibody solution, of 10 mL in volume and at the same concentration as that in the cuvet (0.26 μ*M* expressed in antigen-binding pockets).
3. Adjust the fluorimeter settings to: excitation slit width at 5 nm; emission slit width at 10 nm; excitation wavelength at 290 nm; and emission wavelength at 345 nm.
4. Measure the fluorescence emission of the solution (F; observed intensity of fluorescence in the presence of varying amounts of antigen).
5. Remove from the cuvet 750 μL of antibody/antigen complex solution and replace it with 750 μL of the free antibody solution. Allow the mixture to equilibrate for 1 min and measure the fluorescence emission.
6. Repeat **step 5** for ten consecutive times and record the data. During **steps 5** and **6** the antigen concentration is reduced exponentially over the ten replacements of the antibody-antigen mixture with free antibody, while keeping the antibody concentration unchanged throughout the titration **(Fig. 2)**.
7. Clean the cuvet and fill it with the remainder of the pure antibody solution. Take the measurement of the emission. This value will give you the fluorescence emission of free antibody in solution (Fo). Fit the data using **Equation 1** ***(3,4)*** or variations of it using nonlinear least squares fitting. You can use this equation in any data-plotting program available in your laboratory. We have used this equation in two different programs, MathCad and Origin. If you are using MathCad for fitting your data, then you should perform iterative, manual manipulations to achieve best fit. If you

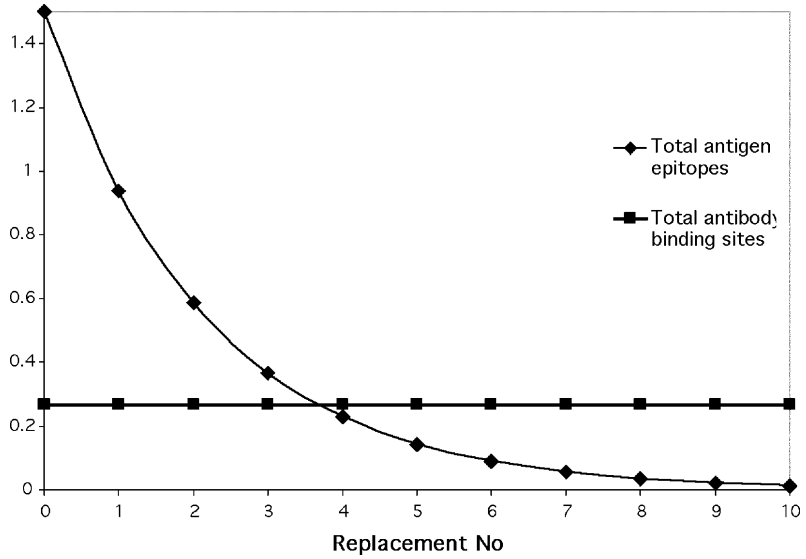

Fig. 2. Relationship between the number of total antigen epitopes and total antigen-binding sites over the course of a flourescence quenching experiment (method one). The antibody concentration remains constant throughout, and the antigen concentration decreases exponentially. Initially there is an excess of antigen epitopes (ratio of 5.8:1, antigen:antibody). At about replacement number 4, there is almost a 1:1 ratio of antigen to antibody. Finally, the antigen concentration decreases to an extent in which there is a large excess of antibody binding sites (ratio of 1:19.6, antigen:antibody). In this way, the occupancy of binding pockets by antigen changes from fully saturated at the start to about 5% occupancy at the end of the experiment.

are using Origin, the program will automatically calculate the values for E1 and K to achieve best fit.

3.2. Using Increasing Amounts of Antigen

A direct titration, in which increasing amounts of antigen are added to an antibody solution and the change in fluorescence is measured at each addition point, can also be employed for the identification of equilibrium binding constants. This method is based on the work of Parker ***(6)***, in which small aliquots of antigen are added to a solution of antibody, and the signal is monitored. The signal is normalized for the dilution effect of adding the antigen to the antibody solution. k_a is then determined by plotting the data. This is a direct titration, in which the first point is that of free antibody and the last point is with the antibody fully saturated by antigen.

1. Prepare 2.5 mL of an antibody solution at a concentration of 0.12 μ*M* in PBS, pH 7.4 (0.018 mg/mL for an IgG antibody). This equals an antigen-binding pocket concentration of 0.24 μ*M*.
2. Place 2.0 mL of the antibody solution in a 3-mL fluorescence quartz~ cuvet of 1-cm cell path length.
3. Adjust the fluorimeter settings to: excitation slit width at 5 nm; emission slit width at 10 nm; excitation wavelength at 290 nm; and emission wavelength at 345 nm.
4. Measure the fluorescence emission of the free antibody solution (Fo; observed intensity of antibody fluorescence in the absence of its antigen).
5. Prepare an antigen solution in PBS at a concentration of 60 μ*M*. The large difference in concentration between the antigen and antibody lessens the need for adding large volumes to the antibody solution in **step 6,** thus minimizing the dilution effect on fluorescence emission.
6. Add 1-μL aliquots of the antigen solution to the cuvet. Mix well and measure the fluorescence emission (F: observed fluorescence in the presence of varying amounts of antigen). Each addition of an antigen aliquot will add a concentration of 0.03 μ*M* of antigen to the antibody solution.
7. It will take you eight additions (experimental points) before you almost reach the one-to-one ratio (antigen epitope to antigen-binding pocket). Make sure you continue your titration to an excess of antigen to antibody, until you achieve several points where the fluorescence does not change (or decreases very slightly because of dilution effects) from measurement to measurement. If your antigen is not as soluble and you have difficulties achieving such concentrations, prepare your antigen in as concentrated a form as possible, making sure to adjust the volumes of the antigen aliquots added to the antibody solution for each measurement.
8. Record the fluorescence emission (F; observed intensity of fluorescence in the presence of varying amounts of antigen) at each step of your titration.
9. Correct for dilution effects resulting from the addition of antigen to your antibody solution. Dilution effects of titrating antigen solution into the antibody solution should be determined by titrating PBS into 2 mL of antibody solution as a control.
10. Plot your data Fo/F vs the antigen concentration, using **Equation 1** (*see* **Subheading 1.**) or any of the available equations for the fitting of binding data, based on nonlinear squares fitting, that would result in the calculation of the association equilibrium constant that represents the binding affinity of your antibody for the given antigen (*see* **Subheading 3.1., step 7**). For fitting data resulting from this method, be sure to consider the dilution in antigen concentration caused by the change in votal volume after each addition of antibody solution.

4. Notes

1. Method one, in which fixed antibody colcentration is used throughout the titration, has an obvious advantage—the concentration of the antibody does not change, thus resulting in more accurate data and equilibrium-constant determination. Therefore, method one should be used, especially if the system is susceptible to changes depending on concentration, or is concentration/volume sensitive in any way.

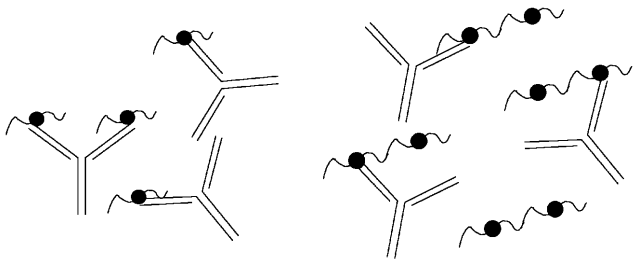

Fig. 3. Monovalent binding of antibody to antigen.

2. Method two presents an advantage over method one because much smaller amounts of antibody are utilized. If sample volume is of the essence, then method two is obviously preferred. However, if at all possible, you would be advised to compare at least once the two methods in your system to determine if and how much concentration/volume changes are affecting your measurements.
3. Both methods described here could be used for any other titrations using optical spectroscopy, such as ultraviolet (UV) or Circular Dichroism (CD) spectroscopy. The only alteration is that these techniques require higher concentrations, and you should adjust your concentrations accordingly to get the required signal. A 10-fold higher antibody concentration (approx 2.3×10^{-6} *M* or 0.35 mg/mL for an IgG antibody) is usually required to obtain a sufficient signal in the UV and CD spectrophotometer in order to perform a titration and obtain equilibrium binding constants. Furthermore, it should be noted here that if you have access to CD spectrophotometer, you can also obtain structural information about your antibody, or changes that occur upon antigen binding, by analyzing the CD spectra in any of the software programs available for CD data analysis.
4. Avoid using Scatchard analysis for fitting your data. It is an outdated and oversimplified representation of the binding event, and can incorporate significant errors in the calculations of the equilibrium binding constants.
5. It is generally accepted that antibody-antigen interactions are classic cases of one-to-one interactions without any cooperativity effects. The antigen-binding site is believed to accommodate a single hapten molecule, and studies also indicate that binding sites in immunoglobulins do not interact in binding of haptens in solution. However, this is not necessarily the case, and avidity can play a part in some experiments in which polymeric antigens with more than one epitope bind to the same antibody's binding sites. This should be taken into account when analyzing the data obtained from such experiments. When one epitope is available per antigen, the association constant reflects that of the single interaction between one epitope and one binding site, with no avidity effects occurring and comparable to association constants described in the literature for antibodies interacting with single epitope molecules. Such an interaction is illustrated in **Fig. 3.** Polymeric antigens with many epitopes may allow two epitopes on the same molecule to interact simultaneously with the same antibody. The possibility of avidity effects on the binding inter-

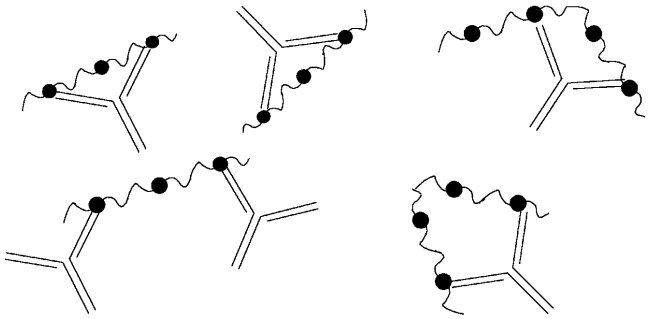

Fig. 4. Monovalent and multivalent binding of polymeric antigens with multiple epitopes.

actions thus exists. It is important to utilize an accurate binding model that would account for such an interaction **(Fig. 4)**. The binding models for mono- and multivalent binding interactions are shown in **Equation 2.** For monovalent interactions, k_a, the equilibrium association constant can be described as:

$$\begin{gathered} Ab + Ag \overset{K_A}{\Leftrightarrow} AbAg \\ K_A = \frac{[AgAb]}{[Ag][Ab]} \end{gathered} \tag{2}$$

Multivalent interactions require a more complex model. The following example shows the interaction between an antibody $Ab_{(2)}$ with two binding pockets and an antigen $Ag_{(3)}$ with three epitopes. (AbAg) represents a binding pocket/epitope complex. Numbers in subscript indicate unbound binding pockets/epitopes.

$$Ab_{(2)} + 2Ag_{(3)} \overset{K_{A1}}{\Leftrightarrow} Ab_{(1)}(AbAg)Ag_{(2)} + Ag_{(3)} \overset{K_{A1}}{\Leftrightarrow} 2(AbAg)Ag_{(2)} \qquad Excess[Ag]$$

Or

$$Ab_{(2)} + Ag_{(3)} \overset{K_{A1}}{\Leftrightarrow} Ab_{(1)}(AbAg)Ag_{(2)} \overset{K_{A1}}{\Leftrightarrow} (2(AbAg))Ag_{(1)} \qquad Excess[Ab]$$

$$K_{A2} > K_{A1}$$

$$K_{A1} = \frac{[Ab_{(1)}(AbAg)Ag_{(2)}]}{[Ab_{(2)}][Ag_{(3)}]} \quad \text{and} \quad K_{A1} = \frac{[(AbAg)Ag_{(2)}]^2}{[Ab_{(1)}(AbAg)Ag_{(2)}][Ag_{(3)}]}$$

Rearrange to substitute out the $Ab_{(1)}$ $(AbAg)Ag_{(2)}$ term to give

$$K_{A1} = \frac{\left[\left(AbAg\right)Ag_{(2)}\right]}{\sqrt{\left[Ab_{(2)}\right]\left[Ag_{(3)}\right]}} \tag{3}$$

Then

$$K_{A2} = \frac{\left[\left(2\left(AbAg\right)\right)Ag_{(1)}\right]}{\left[Ab_{(1)}\left(AbAg\right)Ag_{(2)}\right]} \tag{4}$$

Substitution into **Equation 4** also gives:

$$K_{A2} = \frac{\left[\left(2\left(AbAg\right)\right)Ag_{(1)}\right]}{K_{A1} \times \sqrt{\left[Ab_{(2)}\right]\left[Ag_{(3)}\right]}} \tag{5}$$

There are two equilibrium association constants k_{A1} and k_{A2} depending on the concentration of antigen in the mixture. In antigen excess, there is monovalent binding with each antibody that associates with one epitope on two separate antigen molecules. This gives the association constant which represents the true affinity of the interaction, k_{A1}. In antibody excess, there is bivalent binding of one antibody molecule to two epitopes on the same antigen molecule. This yields the association constant, which incorporates in part the true affinity, as well as avidity, k_{A2}. So when analyzing the data obtained, it is important to do two fittings. For points measured at antigen excess, the graph is produced using the monovalent binding model. For points at antibody excess, the graph is fitted using the bivalent model. k_{A2} is greater than k_{A1} and the association rate is higher for k_{A2}. This is because the second epitope has lost entropy as a result of the complex formed on the same antigen molecule by the first epitope. In effect, the binding of one epitope restricts the conformational space the rest of the antigen molecule can explore before finding a free antigen-binding pocket. The likelihood of associating with the remaining free antigen-binding pocket on the same antibody molecule is much higher because of closer proximity.

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page 442 blank